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Chemical-Inducible Gene Expression Systems for Plants—a Review†

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Abstract: Chemical-inducible expression systems, or 'gene switches', provide an opportunity for the temporal, spatial and quantitative control of genetically engineered traits. This review describes molecular and chemical aspects of several gene switches which have appeared in the literature and a novel unpublished system. Molecular components from plant, bacterial, fungal, insect and mammalian sources have all been utilised to develop gene switches. A brief description of the underlying principle of each approach and some detail of how they perform in transgenic plants is given. Although gene switch systems have utility for fundamental and applied research, particular reference is given to those systems with potential for application in agriculture. © 1998 Society of Chemical Industry

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1 INTRODUCTION

Genetically modified crops have great potential to improve agricultural output. The uptake of this new technology has been rapid, the 2·8 million ha grown in 1996 having increased to 12·7 million ha in 1997.[1] The use of genetically modified crops has already made a significant impact on agrochemical use.[2]

The first wave of biotechnology products in the agricultural market place have mostly been herbicide-, insect- or virus-tolerant varieties. These products contain an introduced DNA cassette comprising a resistance gene under the control of a promoter region which dictates where, when and to what level the resistance gene is expressed. To date the majority of genetically modified crops contain resistance genes under the control of constitutive promoters, for example the 35S promoter from cauliflower mosaic virus (35S CaMV), resulting in the transgene being expressed in all tissue types throughout plant development. Some of the second generation of genetically modified crops will utilise more sophisticated systems of gene regulation. These approaches may include the use of tissue- or organ-specific promoters; for example male sterile plants have been generated using flower-specific promoters.[3]

A further development in this move to a more stringent control of transgene expression is the use of inducible promoters which are activated by the application of a specific chemical stimulus. Chemical-inducible expression systems or 'gene switches' enable the temporal, spatial and quantitative control of genes introduced into plants for the purposes of both academic and applied research. For fundamental research, gene switch systems provide the ability to control the timing and level of transgene expression, which may aid a better understanding of gene function. Highly regulated expression may be of particular relevance if the transgene product leads to deleterious effects.

A range of potential commercial applications includes the regulation of crop-protection genes. The insecticidal CrylA(b) protein of *Bacillus thuringiensis* Berliner has been introduced into transgenic tobacco under the control of the chemical-inducible PR-1a promoter.[4] The

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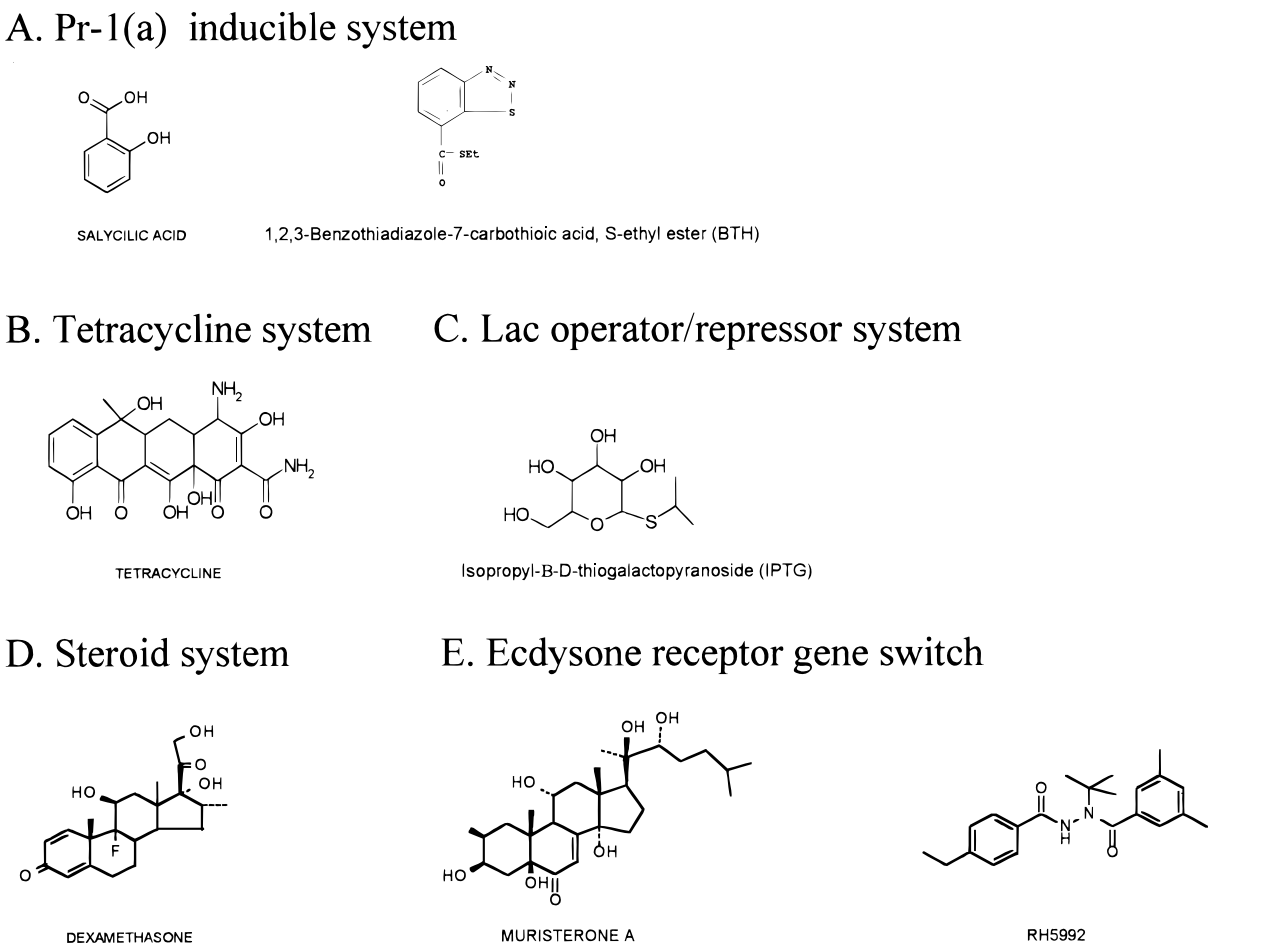

Fig. 1. Compounds used as inducer molecules for chemical-inducible gene expression systems (see text for an explanation of the individual switch systems).

temporal control of insecticidal proteins in transgenic crops may provide opportunities for resistance-management strategies. It has been proposed that chemical-inducible expression systems may allow crop plants to be used as efficient bioreactors to produce recombinant proteins or industrial materials such as thermoplastics.[5] More recently it has been demonstrated that developmental processes in plants can be regulated using chemical-inducible systems. The flowering-time gene CONSTANS has been controlled in a dexamethasone-dependent manner, leading to early flowering in *Arabidopsis thaliana* Heynh.[6]

Chemical-inducible promoters in prokaryotic and lower eukaryotic systems are well developed and in common use.[7] Systems for higher eukaryotes and in particular plants, are relatively poorly developed. A number of approaches to the chemical regulation of transgenes in plants have recently been reviewed and a number of molecular and chemical features were described for an ideal inducible expression system.[8] These include the use of a chemical which is non-phytotoxic, effective at low use-rate, systemic, induces no pleiotropic effects in plants and has applicability to field use. The molecular components should lead to low levels of gene expression in the absence of inducer and increase rapidly to high levels on application of chemical. While a number of inducible expression systems have been described for use in plants and several have been successfully used in laboratory experiments with target genes, none fully meets all these properties described above.

This review article will briefly describe molecular and chemical aspects of a number of inducible systems for plants. Recent developments, particularly those with potential utility for field application will be considered.

2 PLANT PROMOTERS

2.1 Pathogenesis-related promoter PR1-a

A wide range of inducible genes has been identified in plant species. Much of the work in this area has focused on endogenous chemical signals such as phytohormones or responses to pathogen attack or wounding. The corresponding promoter regions of many of these genes do not represent good candidates for chemical-inducible system for plants, as it is not possible to control the

endogenous level of the chemical signal or the external stimulus.

Despite the difficulty of working with endogenous chemical-inducible promoters, a number of systems have been used successfully in transgenic plants. The best-studied system utilises the PR1-a promoter from tobacco which is induced during the systemic acquired resistance response following pathogen attack.[9] PR1-a mRNA levels have been shown to be highly induced by exogenous application of certain chemicals of plant origin such as salicylic acid (SA) or synthetic compounds such as 1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Fig. 1).[8,10,11] BTH is of particular interest as an inducing chemical as it is systemic, used at low field rates and has been recently launched commercially as a plant immunisation compound. Despite PR1-a mRNA levels being highly induced (1000- to 10 000-fold) following SA treatment, the corresponding PR1-a promoter when fused to the β-glucuronidase (GUS) reporter gene only gave approximately 10-fold induction.[5,10] In addition, the use of PR1-a may be limited due to responsiveness to endogenous signals (SA) and external factors such as pathogens, UV-B and pollutants.[8]

2.2 Herbicide safener-inducible gene expression

Detoxification of certain herbicides can be increased by treatment with safeners.[12] One of the primary modes of safener action is the induction of a range of metabolising enzymes, including glutathione *S*-transferases and cytochrome P450s. Several safener-induced cDNA clones have been isolated from maize, including In2-2[13] and GST-27.[14] *Arabidopsis thaliana* has been transformed with a reporter gene construct under the control of the In2-2 promoter.[15] Transgenic seedlings grown in the absence of safener showed no background expression. When grown in the presence of the safener *N*-(aminocarbonyl)-2-chlorobenzenesulfonamide, reporter activity was induced in certain tissues, including the shoot apical meristem, hydathodes and in the root. Plants which were maintained in the presence of safener were retarded in growth. This system clearly requires further optimisation before it has broader utility as a chemical-inducible system for plants. It is interesting to note that the In2-2 promoter could also be induced by the sulfonylurea herbicide chlorsulfuron and branched amino-acids which inhibit acetolactate synthase activity.[15]

3 PROKARYOTIC REPRESSOR–OPERATOR SYSTEMS

One approach used to provide an inducible gene expression system in higher plants is the utilisation of bacterial repressor proteins and the unique operator DNA sequences to which they bind. The use of an evolutionarily distant transcriptional apparatus reduces the risk of induction of host-plant genes and activation at bacterial operators by endogenous transcriptional activators. The system is based on steric hindrance of the polymerase transcription complex by repressor protein bound to the operator in a modified plant promoter (Fig. 2). In the presence of inducer, the repressor dissociates from the operator, allowing access for polymerase and subsequent transcription of the downstream coding sequence. Both the tetracycline and lactose repressor–operator systems from the bacterium *Escherichia coli* (Mig.) Cast. and Chalm (*E. coli*) have been used in plants to control gene expression.[16,17]

3.1 Tetracycline-controllable systems

The tetracycline-inducible promoter system is well characterised and occurs in a number of forms. It has been used successfully in a range of higher eukaryotic systems and represents the best-described system in plants.[7,8] The TetR repressor protein from the Tn10 transposon in *E. coli* and its operator sequence were used in tobacco protoplasts to regulate expression from the 35S CaMV promoter.[18] The inducer tetracycline (Fig. 1) binds to the TetR repressor protein, resulting in a conformational change causing it to be released from the operator and allowing access for polymerase binding and subsequent transcription. Levels of induction of the system in tobacco plants can approach 500-fold[16] in the presence of tetracycline at 1 mg litre^{-1} after 10–14 days. The utility of the Tet system has been demonstrated in separate studies in tobacco[19] and potato plants.[20]

One disadvantage of the repression system model is the requirement to produce high intracellular levels of repressor in order to maintain high occupancy of the operator site(s). The tetracycline-activated (rTA) system removes this requirement and is based on the construction of fusion proteins between strong transcriptional activation domains such as that from the *Herpes simplex* Virus VP16 protein and the Tet repressor protein.[21] In the absence of tetracycline, the fusion

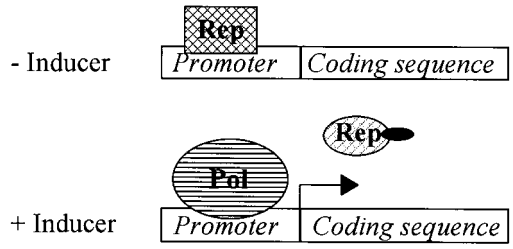

Fig. 2. Schematic representation of a bacterial repressor–operator system. In the absence of inducer the repressor (Rep) binds its target promoter. In the presence of inducer, represented by a black oval, the polymerase transcription complex (Pol) activates transcription, represented by an arrow, of the transgene coding sequence.

protein binds DNA at the operator sequence and activates transcription by interaction with the polymerase protein complex. In the presence of tetracycline, however, transcription is shut off as a result of the release of the fusion protein from the promoter. While the stringency of the tTA-system was superior to that of the tetracycline-inducible system, absolute activity was 3- to 5-fold lower.

Although these tetracycline-based systems have utility in the laboratory, their use in the field is restricted due to the unstable chemistry of tetracycline and the possibility of toxicity to plants grown in conditions of limited drainage. In addition, the tetracycline-inducible system has not been shown to work in all plant species tested and high levels of TetR needed for full repression could not be tolerated without an altered physiology in tomato.[8]

3.2 Lactose-controllable system

The Lac switch system is derived from the *E. coli* lac operon which is activated in response to the presence of lactose and is involved in its import into the cell and metabolism.[22] A lacI gene codes for a repressor protein which binds to the lac operator sequence according to the repressor–operator model (Fig. 2). The natural inducer of the system is allolactose, but active analogues include the synthetic and non-metabolised molecule isopropyl-β-D-thiogalactoside (IPTG; Fig. 1).

The efficiency of the Lac repressor–operator system has been investigated in a plant gene expression system.[17] The lacI coding sequence was placed under the control of the 35S CaMV promoter. A single lac operator sequence was inserted in the maize chlorophyll a/b binding protein (cab) promoter upstream of the β-glucuronidase (GUS) reporter gene. Levels of repression of GUS gene expression in the absence of inducer of 90-fold relative to the unmodified cab promoter levels were seen in tobacco protoplasts. Relief of repression by the addition of IPTG resulted in a 15-fold induction of GUS activity in protoplasts.

The Lac repressor–operator system has not been reported to function in whole plants and in protoplast studies the system required relatively high levels (10 mM) of IPTG for induction. However, the development of other potent non-cytotoxic sugar analogues could give the Lac switch potential for broader utility.

4 LOWER EUKARYOTIC TRANSCRIPTIONAL ACTIVATION SYSTEMS

The use of transcriptional activators and their corresponding response elements from fungal systems reduces the risk of interference between the switch system and the endogenous host-plant transcriptional machinery. To date two systems have been described in the literature.

4.1 Copper-controllable system

A yeast copper-dependent transcriptional activation system has been described which consists of the acel gene encoding a metalloresponsive transcription factor, constitutively expressed from the 35S CaMV promoter, and a GUS reporter gene downstream of a minimal 35S CaMV promoter linked to the ACE1 response elements.[23] Activation of the reporter gene is achieved in the presence of copper, which causes a conformational change in ACE1 and permits binding to the ACE1 response element and subsequent interaction with RNA polymerase. The copper system has been demonstrated in tobacco[23] and *Lotus corniculatus* L. plants.[24] On addition of a 50-μM copper solution to the roots of transgenic tobacco lines containing the system, a 50-fold induction was observed after 24 h. However, tobacco plants left in the copper solution for long periods showed copper-toxicity symptoms.

Copper is used in commercial agriculture primarily as a fungicide on fruit crops such as vines, citrus, pear and peach, and horticultural crops such as coffee and potato. However, in the light of the phytotoxic potential to plants and the possibility of non-specific gene activation through endogenous metal-binding proteins, the applicability of the copper system may not be ideal for all crops.

4.2 Ethanol-controllable system

A recently described transcriptional activation system is based upon the Alc regulon from the fungus *Aspergillus nidulans* (Eidam) Winter[25] which controls its response to ethanol and other related chemicals.[26] The AlcR transcription factor is thought to interact with ethanol or related inducers to bring about the conformational change necessary for DNA binding to specific response elements found in the AlcA promoter (Fig. 3).

In order to assess this two-component system in tobacco, the AlcR coding sequence was expressed from the 35S CaMV promoter and an AlcA promoter fragment containing AlcR response elements was fused to a minimal 35S CaMV promoter linked to the chloramphenicol acetyl-transferase (CAT) reporter gene. The activity of the alc system was assessed relative to a 35 CaMV promoter linked to CAT (p35S : CAT). Tobacco seedlings were treated with an 0·1% ethanol solution applied to the roots for 120 h. Basal levels of CAT activity in the untreated palcR/alcA : CAT seedlings were 1% of that of the induced plants, and in turn these induced levels were 50% of that of the p35S : CAT seedlings (Fig. 4). The utility of the alc system was demonstrated by inducing a yeast cytosolic invertase in

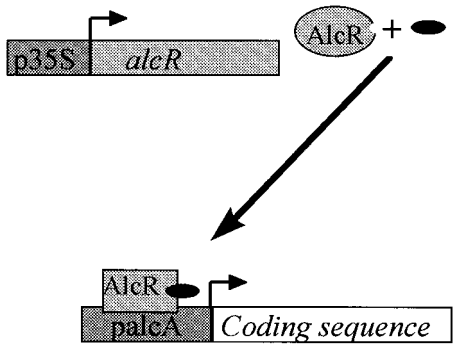

Fig. 3. Schematic representation of the alc system. The AlcR transcription factor is continually synthesised from the CaMV 35S promoter (p35S) and in the presence of ethanol, represented by a black oval, presumably undergoes a conformational change which enables it to bind to the alcA promoter (palcA) and activate transcription, represented by an arrow, of the transgene coding sequence.

tobacco plants which showed a phenotype three days after ethanol treatment.[26]

The alc system has the advantage over previously reported systems in that the inducer, ethanol, is inexpensive, biodegradable and, despite its high volatility, is currently used in agriculture. The alc system itself demonstrates a relatively low basal activity, a rapid and reversible induction and sufficiently high levels of expression to generate phenotypic effects. One potential disadvantage is the risk of induction in situations where ethanol is generated in the plant by severe anoxia. However, experiments involving submergence of tobacco plants in water for four days did not reveal alc induction (Paine, J. A., 1997, pers. comm.).

5 HIGHER EUKARYOTIC TRANSCRIPTION ACTIVATION SYSTEMS

Insect and vertebrate systems for regulation of gene expression may be adapted for plant use, since it is unlikely that these systems will interfere with plant-cell signalling. Hormone receptor systems may be particularly well suited for plant adoption as their molecular biology and chemistry are well understood. The steroid receptors of mammalian cells are a good model for studying the applicability in plants of these heterologous systems.

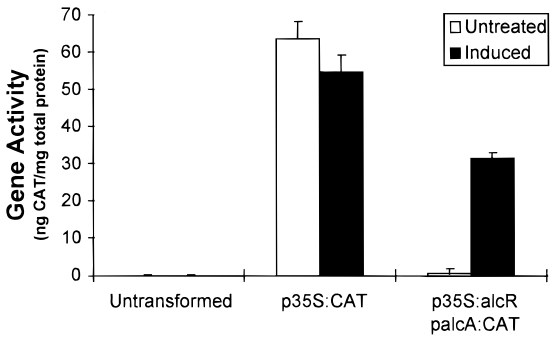

Fig. 4. Comparison of the alc and the CaMV 35S promoter activities. Alc tobacco seedlings (p35S : alcR, palcA : CAT) were tested for CAT protein in comparison with seedlings of a similar plant transformed with the CaMV 35S promoter upstream of the CAT coding sequence (p35S : CAT), and untransformed seedlings. CAT protein levels were tested before and after addition of 0·1% ethanol to the roots of the seedlings. Error bars represent the standard error where $n = 7$.

5.1 Steroid receptors

The steroid receptors are members of the nuclear receptor superfamily and are specific to vertebrate cells.[27] The nuclear receptor superfamily of proteins have six well-defined domains and their activity is ligand-dependent. The receptors have a transactivation domain (A and B) followed by a DNA binding domain (C), a hinge region (D), a ligand-binding domain (E) and a C-terminal region (F). The DNA and ligand-binding domains are the best-conserved regions of the proteins and determine the specificity of the receptors. The steroid receptors differ from other members of the nuclear receptor superfamily in that steroid receptors are compartmentalised in the cytoplasm of the cell in the absence of inducer. As an example, a complex of heat shock proteins, which reside in the cytoplasm, are bound to the ligand-binding domain of the glucocorticoid receptor (GR). Upon addition of inducer, the GR is released from the complex and a conformational change takes place, leading to the receptor entering the nucleus and modulating gene expression (Fig. 5). These properties of steroid receptors make them suitable for control gene expression both at the transcription and post-transcription levels. Steroid receptors, including the GR system, have been used successfully to regulate mammalian gene expression.[28]

In plant cells, expression of GR in tobacco mesophyll protoplasts led to dexamethasone (a glucocorticoid)-dependent reporter gene activity.[29] The GR was introduced into *A. thaliana* plants but failed to induce reporter gene activity upon addition of inducer (dexamethasone), presumably due to weak transactivation potency. A new approach to regulate gene expression transcriptionally has been used, in which the DNA binding domain of Gal4 was fused to the activation domain of the *Herpes simplex* VP16 protein and the ligand-binding domain (Domain E) of GR.[30] Transgenic *A. thaliana* and tobacco plants grown in the presence of dexamethasone induced reporter gene expression. The transcriptional control of gene expression by GR has been shown only in dicotyledonous plants and remains to be tested both in crop species and in monocotyledonous plants.

The GR ligand-binding domain can be used for post-transcriptional as well as transcriptional control of gene expression by using cytoplasmic compartmentalisation as a tool. Several studies have fused transcription

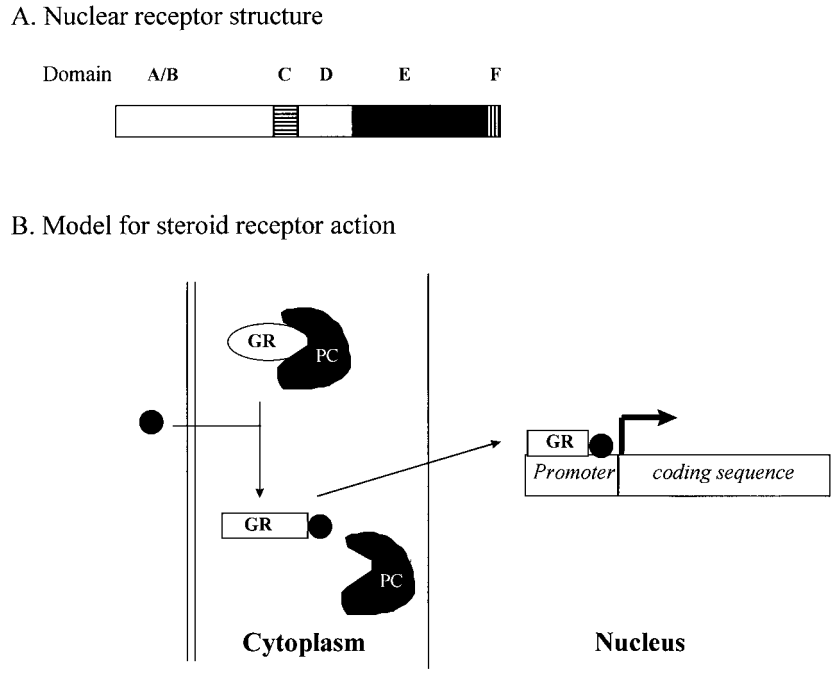

Fig. 5. Schematic representation of the structure and mode of action of the steroid nuclear receptor superfamily. A. A/B represents the transactivation domain, C the DNA binding domain, D the hinge region, E the ligand-binding domain and F the C-terminal domain. B. The glucocorticoid receptor (GR) is bound to a cytoplasmic protein complex (PC), but is released on binding an inducer molecule (dexamethasone), represented by a black circle, by undergoing a conformational change. GR enters the nucleus and promotes transcription, represented by the black arrow, of a target coding sequence from promoters containing GR DNA binding sites.

factors with the GR ligand-binding domain to regulate gene expression post-transcriptionally *via* the application of dexamethasone. Regulation of trichome development in tobacco,[31] leaf morphology in tobacco[32] and flowering time in *A. thaliana*[6] were achieved by the addition of inducer.

Though the use of dexamethasone illustrates the great potential that the steroid receptors have in modulation of plant gene expression, it is clear that steroidal compounds with known vertebrate activity are not suitable for use in the field.

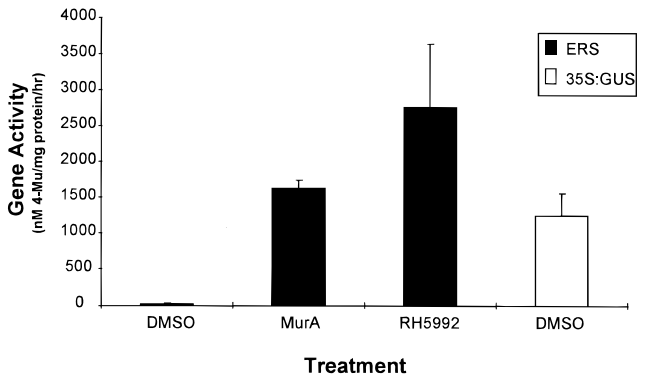

Fig. 6. Chemical inducibility of the ecdysone receptor switch (ERS). Transgenic tobacco seedlings were tested for β-glucuronidase (GUS) protein levels after treatment with 50 μM tebufenozide (RH5992) and 400 μM MuristeroneA (MurA). Levels of GUS activity were found to be comparable to those of the CaMV 35S promoter (35S : GUS). Error bars represent the standard error where $n = 7$. 4-Mu represents 4-methylumbelliferone.

5.2 Ecdysone receptor switch

In order to overcome the difficulties of using steroidal compounds for the regulation of gene expression in transgenic plants, an alternative system was developed using insect ecdysone moulting control receptor sequences. In particular, the ligand-binding domain of the ecdysone receptor of *Heliothis virescens* F. was fused to the transactivation domain of *Herpes simplex* VP16 protein and the GR DNA binding domain. Transgenic tobacco plants, obtained by *Agrobacterium* transformation, led to a population of plants whose reporter gene activity was induced by muristeroneA (a 20-hydroxyecdysone insect moulting hormone agonist; Fig. 1). The plant population was screened using muristeroneA and levels of activation up to 400-fold over the uninduced control were found. MuristeroneA has the disadvantages of being a naturally occurring steroidal compound of limited availability. However, commercial, non-steroidal compounds with ecdysteroid agonist activity have been described in the literature.[33–35] Tebufenozide (RH5992), a member of this family of compounds, has a narrow spectrum of activity and is highly active against some lepidopteran insects.[35] Tebufenozide is commercially available as a suspension concentrate (registered names Confirm and Mimic) for

foliar spray applications to control insect pests in vines (144 g ha^{-1}) and apples (14·4 g ha^{-1}). Transgenic tobacco seedlings grown in the presence of 50 μM tebufenozide were found to activate gene expression to levels comparable to that of the 35S CaMV promoter fused to the GUS reporter gene (Fig. 6). Ten-fold less tebufenozide is required to obtain similar levels of activation compared to muristeroneA. Thus, the ecdysone receptor switch (ERS), following additional characterisation, shows potential for broad application, since the inducer is a commercially available compound with a narrow spectrum of activity capable of high levels of induction.

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